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# Inhibition of Cell Mediated Immune Responses by 8-Methoxypsoralen and Long-wave Ultraviolet Light: A Possible Explanation for the Clinical Effects of Photoactivated Psoralen

VERA B. MORHENN, M.D., CLAUDIA J. BENIKE, B.S., AND EDGAR G. ENGLEMAN, M.D.

*Departments of Dermatology and Pathology, Stanford University School of Medicine, Stanford, California, U.S.A.*

**Human thymus-derived lymphocytes proliferate when cultured with lymphocytes or epidermal cells from unrelated individuals because such cells express HLA-D antigens, which are recognized as foreign by thymus-derived lymphocytes. The current study demonstrates that these responses are inhibited if either the stimulator cells or responder cells are pretreated with the combination of 8-methoxypsoralen and long-wave ultraviolet light. Additional studies revealed that photoactivated 8-methoxypsoralen has a lethal effect on lymphocytes and monocytes but not on the majority of epidermal cells. These observations suggest that the dramatic beneficial effect of PUVA on patients with psoriasis and other skin disorders may be due to a toxic effect on immunocompetent cells in the epidermis, which results in inhibition of cell mediated immune responses.**

The discovery that combined therapy with 8-methoxypsoralen (8-MOP) and long-wave ultraviolet light (UVA) has dramatic beneficial effects on psoriasis has led to increasing clinical use of this treatment, which is known as PUVA [1]. Previous reports indicated that PUVA inhibits cellular DNA synthesis, both *in vitro* [2] and *in vivo* [3], and these observations provided the rationale for the use of PUVA in psoriasis, a disease characterized by epidermal cell hyperproliferation. The hypothesis that PUVA works by inhibiting abnormal cellular proliferation may be valid, but it does not adequately explain the beneficial effects of PUVA in vitiligo [4], alopecia areata [5], mycosis fungoides [6] and lichen planus [7], none of which are characterized by rapid turnover of epidermal cells. Moreover, recent data indicate that the pathogenesis of at least some of these conditions may involve immune mechanisms [8,9], prompting the suggestion that PUVA may work by influencing host immune functions [10].

This study is an effort to determine the effects of PUVA on immunologic reactions elicited by HLA-D antigens. These antigens are expressed by only a few cell types, including lymphocytes [11], monocytes [11], certain bone marrow precursor cells [12] and Langerhans (Lh) cells which represent 2–4% of the cells of the epidermis [13]. To test for the presence of HLA-D antigens, lymphocytes of one individual are incubated for several days with x-irradiated lymphocytes of another donor in the one-way mixed lymphocyte reaction (MLR) [14]. In the epidermal cell-lymphocyte reaction (ELR), epidermal cells are substituted for the irradiated lymphocytes and serve as the stimulating cell population [15,16]. In the ELR, as in the MLR, thymus-derived (T) lymphocytes proliferate in response to foreign HLA-D antigens present on the surface of stimulating cells [17]. In the current study, we assess the effects of PUVA on the capacity of epidermal cells and lymphocytes to stimulate in the ELR and MLR, respectively.

## MATERIALS AND METHODS

### *Lymphocyte isolation and MLR*

Peripheral blood mononuclear leukocytes (PBML) were obtained from healthy volunteers by Ficoll-Hypaque (Ficoll, Sigma Chemical Co., St. Louis, MO; Hypaque, Winthrop Laboratories, New York) density gradient centrifugation [18] of fresh defibrinated venous blood. Where indicated PBML were separated into T lymphocyte enriched and T-cell depleted (non-T) populations with a sheep erythrocyte rosetting technique as described [19]. In the MLR, $5 \times 10^4$ responder PBML were cultured with $5\times 10^4$ x-irradiated allogeneic stimulator PBML in 0.2 ml of RPMI 1640 medium (Microbiological Associates, Walkerville, MD.) supplemented with 10% heat inactivated pooled human serum, and 2 mM L-glutamine (complete RPMI). After 6 days of culture in 10% $CO_2$ at 37°C, $^3$H-thymidine (New England Nuclear, Boston, Mass.) was added (1 $\mu$Ci/well), and the cells were harvested 18 hr later.

### *Epidermal Cell Preparation and ELR*

As described previously [20], epidermal cell suspensions were prepared from keratotomed cadaver skin by exposure to 0.3% trypsin (Microbiological Associates, Walkerville, Md.) in 0.15 M NaCl, 5.4 mM KCl, 0.1% glucose at pH 7.6. After 40 min at 37°C, the cells were resuspended in complete RPMI 1640 medium and filtered through 100 gauge wire mesh. In the ELR, $5 \times 10^4$ epidermal cells were co-cultured with $5 \times 10^4$ allogeneic lymphocytes under conditions otherwise identical to those of the MLR.

### *PUVA Treatment*

Treatment of cells with PUVA consisted of a 1 hr incubation at 37°C with varying concentrations of 8-MOP. Two ml aliquots of cells were then placed in a 35 × 10 mm plastic Petri dish (Lux Scientific, Newbury Park, Ca.) covered with a 4-mm thickness of window glass, which absorbs light of wavelength less than 320 nm. A high-intensity lamp (Sylvania tube #GBO-3824-231, Sylvania Corp., Danvers, Ma.) was used to irradiate the cells with 1.8 J/cm$^2$ of near ultraviolet light (365 nm) in a light box used for treatment of patients. After treatment, the cells were centrifuged at 300 ×*g*, resuspended in the medium described above, and immediately aliquoted into microtiter wells for culture. The incident UVA-light was monitored using the radiometer supplied with the light box. This meter measures in the range between 320-380 nm [21]. The Petri dishes were positioned on top of a foot stool placed on the floor of the box and always irradiated at the same relative distance from the light bulbs. To measure the filtration effect of the Petri dish covers, the medium and the window glass, a portable photometer (H. Waldmann) which measures between 320-400 nm (H. Schaefer, personal communication) was used. The cover of the Petri dish, 2 ml of medium and the window glass each decreases the incident dose of UVA about 10%. At the settings used, the dose of UVA reaching the cells was 1.8 J/cm$^2$. About 1% of the light output is in the wavelengths between 290-320 nm (UVB). Using a radiometer (International Light), we determined that the window glass decreases the incident UVB by 10% and the Lux Petri dish covers further decrease the UVB by 10-

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Reprint requests to: Vera B. Morhenn, M.D., Stanford School of Medicine Department of Dermatology, Stanford, California 94305.

Abbreviations:
ELR: epidermal cell-lymphocyte reaction
MLR: mixed lymphocyte reaction
8-MOP: 8-methoxypsoralen
PBML: peripheral blood mononuclear leukocytes
PUVA: 8-methoxypsoralen combined with long wave ultraviolet light
UVA: long-wave ultraviolet light
UVB: short-wave ultraviolet light

fold. Thus for every J/cm$^2$ of UVA the cells receive less than 1 mJ/cm$^2$ UVB. The lamps were prewarmed for at least 5 min before use on the cells.

*X-Irradiation of Cells*

Where indicated stimulator cells were irradiated in a $^{137}$Cesium irradiator (Mark I, irradiator, JL Shepherd, Glendale, Ca.) with a dose of 3,000 rads to abolish their capacity to proliferate and to make the reaction unidirectional.

*Assesment of Cell Viability*

Cells were treated as indicated, centrifuged, resuspended in complete RPMI medium aliquoted into plastic test tubes (Falcon, Oxnard, CA) and incubated at 37°C. At intervals aliquots were removed and viability of epidermal cells and T and non-T lymphocytes was determined as described [22]. Briefly, 1 ml of cell suspension was incubated with .1 ml of .01% fluroescein diacetate, incubated for 5 min in the dark, washed with phosphate buffered saline (PBS) and resuspended at $2 \times 10^6$ cells/ml. One drop of this suspension was placed on a slide with 5λ of ethidium bromide (0.04% in PBS) and examined immediately with a fluorescence microscope. Viable cells show green fluorescence whereas dead cells stain red.

## RESULTS

Table I demonstrates that epidermal cells are potent stimulators of allogeneic lymphocytes. As shown, stimulation in the ELR was prevented by PUVA pretreatment of the epidermal cells but not by 3,000 rads x-irradiation. The effect of PUVA treatment was dependent on the concentration of 8-MOP used, and at the highest concentration tested (2.5 μg/ml) the reaction was completely blocked. Pre-incubation of epidermal cells with 8-MOP but without subsequent exposure to UVA, had only a slight inhibitory effect on the ELR. Exposure to UVA alone had a similar effect. The results of assays using $^3$H-thymidine incorporation as a measure of proliferation were paralleled by the number of blast cells counted microscopically (data not shown). Comparable results have been obtained in 6 experiments.

PUVA also prevented stimulation by lymphocytes in the MLR, and representative data are shown in Table I. In this set of experiments, PBML that had been treated with 3,000 rads x-irradiation were either placed in culture immediately or further treated as indicated prior to culture with allogeneic responder lymphocytes. Again, both 8-MOP and UVA were required for complete blockade, and inhibition was observed at the same concentration of 8-MOP which blocked stimulation of epidermal cells in the ELR.

In addition to its effect on stimulator cells, PUVA prevented responder lymphocytes from proliferating (Table II). In these reactions, all the stimulator cells were inactivated with 3,000 rads x-irradiation and the responder cells were treated as indicated. As can be seen, even the milder PUVA treatment prevents the proliferative response of allogeneic lymphocytes. Thus, the concentration of photoactivated 8-MOP required for complete inhibition of response was less than the concentration which abrogated the stimulatory capacity of lymphocytes or epidermal cells. Further, 1.8 J/cm$^2$ UVA alone inhibited the proliferation of responder lymphocytes to 75% of control whereas this treatment decreased the stimulatory capacity of epidermal cells and lymphocytes only slightly (10% and 30%, respectively).

One possible explanation for these results is that PUVA has a lethal effect on the cells stimulating and responding in the ELR and MLR. To assess this possibility, the viability of PUVA treated epidermal cells and lymphocytes was monitored by staining viable cells with fluorescein diacetate and counterstaining dead cells with ethidium bromide [22]. As shown in the Figure, the viability of epidermal cells was only minimally affected by PUVA. In contrast, PBML were sensitive to a variety of treatments, including PUVA, UVA and x-irradiation. Purified T cells were sensitive to all of the treatments, whereas non-T cells (primarily B-lymphocytes and monocytes) were particularly sensitive to PUVA and less sensitive to other treatments. Thus, more than 80% of initial lymphocyte populations were dead 48 hours after receiving the high dose of PUVA. Two experiments were done on cells from different donors and similar results were obtained each time.

## DISCUSSION

In this report we have shown that immunologic reactions elicited by HLA-D antigens are inhibited by PUVA. Inhibition by PUVA of the MLR has recently been reported [23]. The current work shows that the ELR as well as the MLR is inhibited by PUVA and demonstrates that PUVA affects both stimulator and responder cells. This inhibition may be explained by a second observation, that PUVA has a lethal effect on the cells stimulating or responding in the reactions studied, that is, lymphocytes, monocytes and possibly Lh cells of the epidermis. On the other hand, keratinocytes which represent the vast majority of epidermal cells are relatively resistant to PUVA treatment. Since keratinocytes are usually exposed to a broad spectrum of UV irradiation from the sun, the relative resistance of these cells to the toxic effects of UV light is not surprising.

The intracellular mechanism of the PUVA effect is unknown. PUVA treatment of fibroblasts and epidermal cells causes inhibition of cellular DNA synthesis [2,3]. Therefore, the observed inhibition of the ELR and MLR may be mediated by inhibition of DNA synthesis in the stimulator cells. Although it is standard procedure in the unidirectional MLR to render stimulator cells incapable of detectable DNA synthesis by treating them with either x-iradiation [24] or mitomycin C [25], a low level of DNA synthesis undetectable by standard methods may be required for stimulation. Alternatively, PUVA either alone or in combination with the small amount of contaminating UVB may have an effect similar to that previously reported for UVB, which is thought to inhibit stimulation in the MLR by preventing RNA synthesis [26]. A final possibility

TABLE I. *Effect of PUVA on the capacity of epidermal cells and lymphocytes to stimulate allogeneic lymphocytes*

| Treatment of stimulator cells[a] | Responses of lymphocytes to allogeneic epidermal cells | | Responses of lymphocytes to allogeneic lymphocytes | |
|---|---|---|---|---|
| | Donor A | Donor B | Donor C | Donor D |
| | Δ *cpm* ± *SE*[b] | | Δ *cpm* ± *SE*[b] | |
| None | 47,150 ± 5052 | 43,069 ± 2630 | — | — |
| 3,000 rads x-irradiation | 34,588 ± 3903 | 30,627 ± 4177 | 20,239 ± 1642 | 25,458 ± 2017 |
| 0.5 μg/ml 8-MOP plus 1.8 J/cm$^2$ UVA | 18,331 ± 3380[c] | 5,145 ± 825[d] | 578 ± 38[d] | 138 ± 85[d] |
| 2.5 μg/ml 8-MOP plus 1.8 J/cm$^2$ UVA | 198 ± 27[d] | 44 ± 11[d] | 1,044 ± 155[d] | 870 ± 218[d] |
| 2.5 μg/ml 8-MOP | 29,004 ± 1325 | 33,647 ± 3566 | 16,316 ± 2688 | 19,203 ± 2430 |
| 1.8 J/cm$^2$ UVA | 31,708 ± 4671 | 26,330 ± 1594 | 14,118 ± 904 | 18,325 ± 1649 |

[a] Epidermal cells were treated as indicated. The stimulator lymphocytes were treated with 3,000 rads x-irradiation and then either immediately put into culture or further treated with .5 μg/ml photoactivated 8-MOP, 2.5 μg/ml photoactivated 8-MOP, 2.5 μg/ml 8-MOP or 1.8 J/cm$^2$ UVA.

[b] Response of lymphocytes cultured alone (475 cpm) subtracted to yield Δcpm.

[c] $p < .01$ by Student *t*-test.

[d] $p < .001$ by Student *t*-test.

TABLE II. *Effect of PUVA on the capacity of lymphocytes to respond in the MLR*

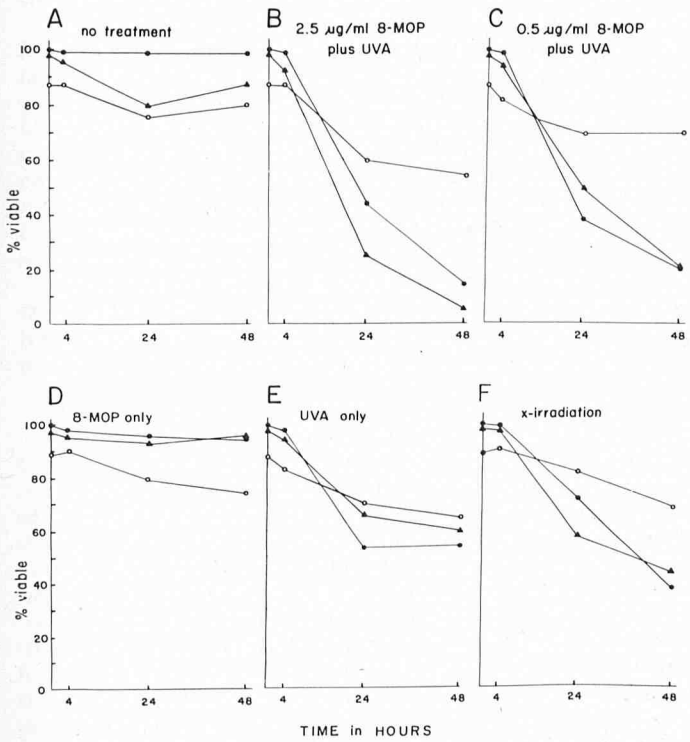
| Treatment of responder cells[a] | Proliferation of responder lymphocytes to allogeneic x-irradiated lymphocytes | |
|---|---|---|
| | Donor E | Donor F |
| | Δ *cpm* ± *SE*[b] | |
| None | 24,463 ± 912 | 22,458 ± 2,081 |
| 3,000 rads x-irradiation | 471 ± 56[c] | 203 ± 18[c] |
| 0.5 μg/ml 8-MOP plus 1.8 J/cm² UVA | 375 ± 73[c] | 711 ± 142[c] |
| 2.5 μg/ml 8-MOP plus 1.8 J/cm² UVA | −213 ± 81[c] | −36 ± 12[c] |
| 2.5 μg/ml 8-MOP alone | 21,806 ± 2133 | 18,401 ± 1,492 |
| 1.8 J/cm² UVA alone | 6,410 ± 1160[d] | 6,017 ± 1,884[d] |

[a] Responder lymphocytes were pretreated as indicated, cocultured with allogeneic donor (E, F) lymphocytes and proliferation measured at day 7.

[b] Proliferation of responder lymphocytes cultured alone (912 cpm) subtracted to yield Δcpm.

[c] $p < .01$ by Student *t*-test.

[d] $p < .001$ by Student *t*-test.




Viability of epidermal cells, T and non-T lymphocytes following various forms of treatment. *Brackets* indicate standard error of the mean. Populations of epidermal cells, T cells and non-T cells were treated as indicated. In *D*, the cells were treated with 2.5 μg/ml 8-MOP only. Following treatment, viability was determined at 0, 4, 24 and 48 hr by staining cells with fluorescein diacetate and ethidium bromide (○——○ epidermal cells, ●——● T lymphocytes, △——△ non-T lymphocytes).

is that the effects observed here are not related to a primary central metabolic insult, but may be the result of interaction between PUVA and the cell surface membrane, possibly involving alteration of HLA-D antigens. It should be noted that alteration of the cell membrane might be an indirect result of any of these proposed mechanisms.

Regardless of the precise subcellular site of PUVA action, the data indicate that lymphocytes, monocytes, and possibly Lh cells are more susceptible to this treatment than the predominant epidermal cell, the keratinocyte. Since the Lh represents such a small fraction of the total epidermal cell population, a selective lethal effect of PUVA on this cell type would not be apparent from our viability studies on total epidermal cells. Recent evidence suggests that the majority of Lh cells in the epidermis are bone marrow derived [27] and that they probably play a vital role in the afferent limb of the immune response leading to contact sensitization [28]. Moreover, PUVA treatment abolishes the delayed hypersensitivity response at sites previously sensitized to an antigen [29] which indicates that Lh cells may be inactivated or killed by PUVA therapy. It is possible, therefore, that the clinical effectiveness of PUVA may be due to its toxic effect on Lh cells or other immunocompetent cells in the skin, which results in inhibition of cell-mediated immune functions. Proof of this hypothesis of PUVA action would be of considerable interest since it would confirm that the immune system has a direct role in the pathogenesis of psoriasis as well as other skin disorders.

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